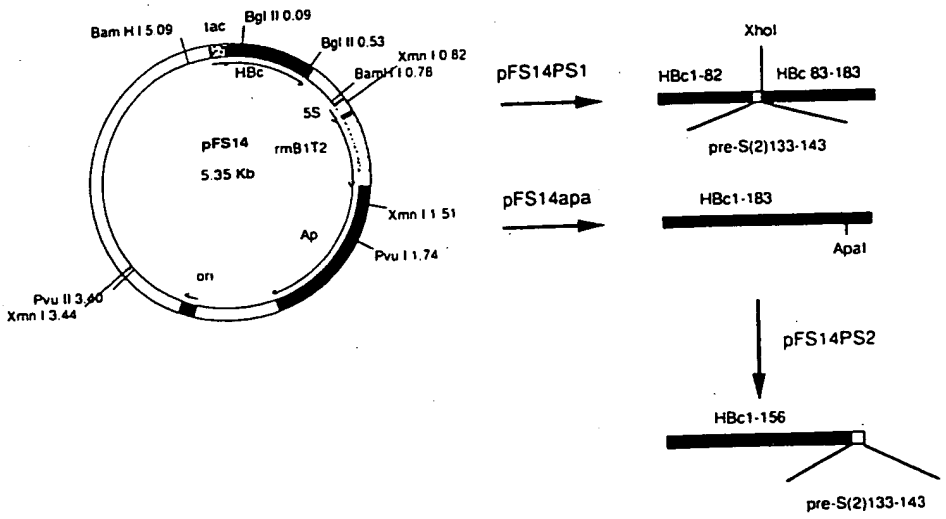

Figure 1
Structure of expression plasmid pFS14 and derivatives pFS14PS1, pFS14apa, and pFS14PS2. For details, see text.

restriction sites were bridged with a pair of complementary asymmetric synthetic oligonucleotides of the *ayw* sequence. The sequence of the coding oligonucleotide was 5′-AAT TAT GGA CAT-3′. The construct places the core gene ATG at position +11 relative to the Shine-Dalgarno sequence of pKK223 (pFS14, Fig. 1) and contains the complete nucleotide sequence of the *ayw* HBc gene. When induced with IPTG in *E. coli* JM105, this plasmid directs the synthesis of the 21-kD HBcAg immunoreactive with anti-HBcAg antibodies in immunoblots (see Fig. 3b in Schödel and Will 1989) and in ELISA of up to approximately 1% of whole-cell proteins, as judged from Coomassie-stained polyacrylamide gels (not shown). The particulate nature of HBcAg produced from pFS14 has been demonstrated by characteristic sedimentation properties in sucrose gradients, by banding in cesium chloride density gradients, and by electron micrography (data not shown). Immunization with recombinant HBV core particles purified by cesium chloride density gradient centrifugation induced a protective immune response against challenge with woodchuck hepatitis virus (WHV) in two of three woodchucks (F. Schödel et al., in prep.). We therefore assume that amino acid sequences conserved between HBV and WHV mediate protective immunity (for an amino acid comparison between hepadnavirus core proteins, see Schödel et al. 1989). An interesting candidate is the region between amino acids 120 and 140 of HBcAg, which is highly conserved between WHV and HBV, encompasses two T-cell sites recognized in the murine model by mice of H-2^b and H-2^s haplotype, and is also immunogenic at the B-cell level as a free peptide (Milich et al. 1987a).

Construction of Core/Pre-S2 fusions

Amino-terminal Fusions

We first set out to construct expression vectors that would direct the synthesis of core proteins with the pre-S2 *ayw* (amino acids 133–140) sequence fused to the amino termi-

nus of HBcAg. Using 5′-degenerate oligonucleotides in polymerase chain reactions, we fused the sequence coding for amino acids 133–140 of the HBV pre-S2 region as a tandem repeat to the 5′ end of the core gene in a *trc* promoter vector and as a single repeat in a *tac* promoter vector. With both vectors, stable core or pre-S2 reactive fusion protein could not be detected in the appropriate *E. coli* hosts (not shown), although promoter and pre-S2/core gene sequences were not mutated, as verified by dideoxy sequencing.

Internal Fusion

A recent model for the HBcAg particle structure, based primarily on homologies with RNA virus nucleocapsid particles for which structural information is available, suggests that the site of a 39-amino-acid insertion in the core protein of duck HBV, a related hepadnavirus, is exposed on the surface (Argos and Fuller 1988). To achieve translational fusion of the pre-S2 *ayw* (amino acids 133–143) sequence within the HBcAg sequence, we digested pFS14 partially with *Xba*I, inserted a pair of complementary synthetic oligonucleotides (coding strand 5′-CTA GGG ACC GCG TGT TCG TGG TCT GTA CTT CCC GGC TCG GAG-3′) coding for the pre-S2 sequence 3′ of the sequence coding for HBcAg up to amino acid position 82, and introduced at the same time a unique *Xho*I site at the 3′ end of the insert (pFS14PS1, Fig. 1). JM105 was transformed, and colonies were screened for expression of pre-S2 epitopes using a monoclonal antibody (Okamoto et al. 1985) kindly provided by M. Mayumi. Plasmid DNA was isolated from positive colonies, the inserted sequence was verified by dideoxy sequencing, and the resultant fusion protein was analyzed by immunoblotting (Fig. 2, left). We induced and lysed JM105 (pFS14PS1) under nondenaturing conditions and separated soluble proteins on a Sepharose 4B column. Anti-core reactivity identical to the chromatographic behavior of particles expressed from pFS14 was found only in the exclusion volume (not shown). Thus, we assume that the internal fusion does not interfere with particle formation. Whether hybrid particles from pFS14PS1 carry the pre-S2 epitopes on their surface is unresolved. Raw bacterial lysates do not react with anti-pre-S2 monoclonal antibodies 4408 or 5520 (Okamoto et al. 1985), but these lysates do react with rabbit anti-pre-S2 (120–145) (provided by R. Neurath). As expected, most monoclonal antibodies against HBcAg do not recognize products from pFS14PS1, since the major epitope on core particles is interrupted by the insertion.

Carboxy-terminal Fusion

As a third site for fusing pre-S2 sequences to HBcAg, we chose the carboxyl terminus of HBcAg. For this purpose, a unique *Apa*I site was introduced at HBV nucleotide position 461 (for nucleotide position references, see Schödel and Will 1989) in an HBV subfragment cloned into a plasmid vector suitable for site-directed mutagenesis (Stanssens et al. 1989). The T residue at position 463 of the authentic *ayw* HBV sequence was replaced by a G, resulting in plasmid pMHM7. The serine at HBcAg amino acid position 155 is thereby changed to an alanine. The core gene *Bgl*II fragment of pFS14 was replaced by the corresponding *Bgl*II fragment from pMHM7 to give plasmid pFS14apa (Fig. 1). Using the unique *Apa*I site in pFS14apa, heterologous gene fragments can be inserted that result in fusion proteins with HBcAg carboxy-terminal to amino acid position 156. In these fusion proteins, the arginine-rich nucleic-acid-binding domain at the extreme carboxyl terminus of HBcAg is deleted, which may be an advantage for prospective use as a vaccine, and seems not to interfere with particle formation. We have inserted a pair of synthetic oligonucleotides (coding strand 5′-CCG GAC CCG CGT GTT CGT GGT CTG TAC TTC CCG GCT TA-3′) specifying again an HBV pre-S2 se-

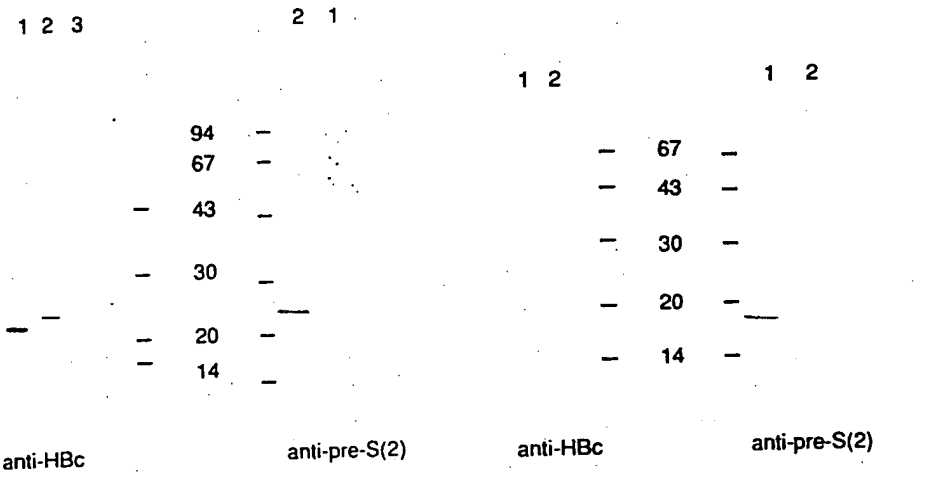

Figure 2
Immunoreactivity of hybrid HBc/pre-S2 fusion proteins from pFS14PS1 (*left*) and pFS14PS2 (*right*) expressed in *E. coli* JM105. Bacterial overnight cultures without plasmid (JM105) or with the indicated plasmid were diluted 1:10 in Standard 1 medium, grown to a density of approximately 0.8 OD_{600}, and induced at 37°C by 1 mM IPTG for 1 hr. For conditions of electrophoresis (on 15% SDS-PAGE) and immunoblotting, see Schödel and Will (1989). Anti-HBc is a polyclonal rabbit serum raised against a purified MS2-HBcAg fusion expressed in *E. coli*, and anti-pre-S2 is a monoclonal antibody (hybridoma 5520) kindly provided by M. Mayumi (Okamoto et al. 1985). The left blot in the left panel is labeled with ^{125}I-protein A. All of the other blots are with alkaline phosphatase-coupled goat anti-mouse or anti-rabbit IgG (H+L) (Medac) using NBT/BCIP as substrate.

quence (133–143), resulting in expression plasmid pFS14PS2 (Fig. 1). A fusion protein with core and pre-S2 reactivity of the expected molecular weight was observed in immunoblots from induced JM105 (pFS14PS2) raw extracts (Fig. 2, right). Nondenatured proteins of the JM105 (pFS14PS2) whole-cell bacterial lysate separated on Sepharose 4B again displayed anticore reactivity only in the void volume identical to that of pFS14 and pFS14PS1 (not shown), which we interpret as evidence for particle formation. The fused pre-S2 epitopes are also detected by anti-pre-S2 monoclonal antibodies 4408 and 5526, as well as rabbit antipeptide pre-S2 (120–145) in nondenaturing ELISAs of bacterial lysates, strongly suggesting their accessibility on the particle surface.

SUMMARY AND CONCLUSION

We have constructed a prokaryotic expression vector directing the synthesis of HBcAg of the authentic *ayw* sequence (pFS14) in *E. coli*. Gene fragments coding for immunodominant conserved B-cell epitopes of the pre-S2 region of HBV surface antigens were fused to the amino terminus, to internal HBcAg sequences, and to the carboxy terminus of HBcAg. Amino-terminal fusions did not lead to expression of stable fusion proteins. Both internal and carboxy-terminal fusions, the latter also removing the HBcAg nucleic-acid-binding region, led to expression of stable fusion proteins with dual reactivity in immunoblots. B-cell epitopes fused to the carboxyl terminus of HBcAg were accessible to antibodies in nondenaturing assays and are thus probably located at the surface of HBcAg particles. The immunoreactivity of purified hybrid HBcAg/pre-S2 particles is cur-

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rently being tested, and it will be especially interesting to determine whether the fused B-cell epitopes will acquire T-cell-independent immunogenicity. In addition, size limits and accessibility of different internal inserts as well as carboxy-terminal fusions will be determined. The existence of HBcAg hybrids with the same or different heterologous epitopes at separate amino acid positions raise the possibility of creating mixed particles and of studying their immunogenicity.

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